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<p>(54) Title: METHODS FOR INCREASING T CELL PROLIFERATION</p> <p>(57) Abstract</p> <p>The invention provides methods and compositions for increasing T cell proliferation using tryptophan enhancing agents. T cell proliferation can be increased <i>in vitro</i> by addition of tryptophan enhancing agents to T cell culture, or <i>in vivo</i> by administration of tryptophan enhancing agents. Also provided are methods for diagnosing and treating disorders characterized by constitutive expression of indoleamine-2,3-dioxygenase. Compositions and apparatus relating to the methods also are provided.</p>																					
<p style="text-align: center;">GROWTH CURVE OF MZ2-CTL-82/30</p> <table border="1"> <caption>Data points estimated from the Growth Curve graph</caption> <thead> <tr> <th>Days of Culture</th> <th>82/30 + 1-METHYL-TRYPTOPHAN (Millions Cells)</th> <th>82/30 (Millions Cells)</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>1</td> <td>1</td> </tr> <tr> <td>7</td> <td>5</td> <td>4</td> </tr> <tr> <td>14</td> <td>15</td> <td>12</td> </tr> <tr> <td>21</td> <td>35</td> <td>22</td> </tr> <tr> <td>28</td> <td>40</td> <td>25</td> </tr> </tbody> </table>				Days of Culture	82/30 + 1-METHYL-TRYPTOPHAN (Millions Cells)	82/30 (Millions Cells)	0	1	1	7	5	4	14	15	12	21	35	22	28	40	25
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METHODS FOR INCREASING T CELL PROLIFERATION

Background of the Invention

In vitro culture of T lymphocytes has become an essential tool of immunology.

- 5 Among other things, it has allowed the characterization of T cell epitopes present on cells infected by various pathogens or on tumor cells, and the *in vitro* study of T cell biology in all its aspects. T lymphocyte culture is also being used in therapeutic applications, including clinical trials based on the infusion of large numbers of T lymphocytes expanded *in vitro* (for a review see Yee et al., *Curr. Op. Immunol.* 9:702-708, 1997).
- 10 However, T cell culture is a difficult procedure. For reasons which are not understood, only a limited number of cytolytic T lymphocyte (CTL) clones can grow *in vitro* and most of those clones will do so only for a limited number of divisions, or at a very slow pace.
- 15 Accordingly, there is a need for improved cell culture procedures or components that increase the efficiency of T cell culture. There also is a need for methods and compositions to improve the diagnosis and therapy of cancer.

Summary of the Invention

- It now has been discovered that increasing tryptophan in T cell cultures, by inhibiting enzymes which catabolizes tryptophan, such as indoleamine 2,3-dioxygenase (IDO), or by adding tryptophan to the culture, increases the proliferation of T cells by increasing the rate of proliferation and/or extending the number of divisions of the T cells in culture. In addition, increasing tryptophan in T cell cultures also enhances the lytic activity of the T cells grown in culture. Compositions and apparatus for increasing proliferation of T cells *in vitro* and/or the number of divisions of T cells *in vitro* and/or lytic activity are also provided.
- 25 Thus, according to one aspect of the invention, methods for increasing proliferation of cells are provided. The methods include growing the cells in the presence of an effective amount of one or more tryptophan enhancing agents to increase the proliferation of the cells. Proliferation of cells is determined preferably by measuring the number of cells in a population before and after growing the cells. Increasing cell proliferation means increasing 30 the number of cells by at least about 10% relative to the number of cells that are present in a parallel control population of cells that are subjected to the same conditions as the tryptophan enhancing agent treated population with the exception that the control population is not contacted with the tryptophan enhancing agents. Preferably, the number of cells are increased at least about 50%, more preferably 2-fold, yet more preferably 4-fold, still more preferably,

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10-fold and, most preferably, at least 20-fold relative to the number of cells that are present in the parallel control population. In certain embodiments, the cells are not macrophages or trophoblasts.

Preferably the cells are T cells. For *in vivo* methods of increasing T cell proliferation,

- 5 growing the T cells means proliferating the T cells *in vivo*, e.g., by administering a population of T cells to a subject. *In vivo* methods for increasing proliferation of T cells can be tested in animal models to establish effective amounts of tryptophan enhancing agents for increasing T cell proliferation. For *in vitro* methods of increasing T cell proliferation, growing the T cells means proliferating the T cells *in vitro*, e.g., by culturing according to standard cell culture
10 methods. *In vitro* proliferation of T cells is readily tested by standard methods of measuring cell proliferation including cell enumeration and metabolism tests.

Preferred tryptophan enhancing agents are IDO inhibitors, including L-methyl-DL-tryptophan, β -(3-benzofuranyl)-DL-alanine and β -[3-benzo(*b*)thienyl]-DL-alanine, most preferably L-methyl-DL-tryptophan. The tryptophan enhancing agent preferably is not
15 tryptophan.

In another aspect of the invention, methods for increasing activity of T cells are provided. The methods include culturing the T cells in the presence of an effective amount of one or more tryptophan enhancing agents to increase the activity of the T cells. In these methods, T cell activity is increased at least about 10%, preferably at least about 25%, more
20 preferably at least about 50%, and most preferably at least about 100%.

According to another aspect of the invention, an apparatus is provided for practicing the methods of the invention. The apparatus includes a container, an inhibitor of IDO contained therein or attached thereto and at least one T cell. Preferably, the container is a sterile conventional cell culture container, known to those of ordinary skill in the art,
25 including e.g., stirring flasks, stirred tank reactors, air lift reactors, suspension cell reactors, cell adsorption reactors and cell entrapment reactors, petri dishes, multi well plates, micro titer plates, test tubes, culture flasks, and bags. The inhibitor of IDO is contained in the container in soluble or immobilized form or directly attached to the internal surface of the container. In addition to containing the immobilized or soluble IDO inhibitor, the container optionally
30 includes one or more growth media components for cell culture. Such components are known to those of ordinary skill in the art. In other embodiments, tryptophan is contained in the culture container. In still other embodiments tryptophan and one or more inhibitors of IDO are contained in the culture container. Additional cell culture components such as growth

medium, serum and other cell types including feeder cells and target cells or antigen presenting cells also can be included. The container does not contain macrophages or trophoblasts.

According to yet another aspect of the invention, a kit for stimulating the proliferation
5 of T cells in culture is provided. The kit contains the apparatus described above and instructions for using the apparatus to stimulate proliferation of T cells *in vitro*.

In another aspect, the invention provides a growth medium for the culture of cells including a tryptophan enhancing agent. Preferably the growth medium contains nutrients for the culture of T cells.

10 According to still a further aspect of the invention, a T cell culture is provided. The T cell culture includes at least one T cell, growth medium and a tryptophan enhancing agent. In some embodiments the culture does not contain macrophages or trophoblasts.

The invention also provides adjuvant compositions comprising the tryptophan enhancing agents described herein and a pharmaceutically acceptable carrier. Use of the compositions
15 described herein in the preparation of a medicament for increasing T cell proliferation and/or activity in a subject also is provided.

Thus, an immune response modulation composition is provided in accordance with the invention. The composition includes an amount of a tryptophan enhancing agent effective to increase local tryptophan concentrations in the presence of constitutively expressed
20 indoleamine 2,3-dioxygenase. The composition also includes a pharmaceutically acceptable carrier. In some embodiments, the tryptophan enhancing agent is an inhibitor of indoleamine 2,3-dioxygenase, preferably 1-methyl-DL-tryptophan. In other embodiments, the immune response modulation composition further includes one or more immune modulators selected from the group consisting of antigen-specific T lymphocytes, peptide antigens, antigenic
25 proteins and nucleic acids encoding peptide antigens. These immune response modulation compositions preferably also include an adjuvant. Preferred adjuvants include monophosphoryl lipid A (MPL); saponins including QS21, DQS21, QS-7, QS-17, QS-18, and QS-L1; DQS21/MPL; incomplete Freund's adjuvant; complete Freund's adjuvant; montanide; and water-in-oil emulsions prepared from biodegradable oils.

30 In yet another aspect of the invention, methods for treating cancer cells which have evaded or have the potential to evade T cell-mediated cytosis are provided. The methods include administering to a subject in need of such treatment an amount of a tryptophan enhancing agent effective to increase T cell-mediated cytosis of the cancer cell. In certain

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embodiments, the cancer cells constitutively expresses indoleamine 2,3-dioxygenase. Other methods include contacting the cancer cells with an amount of a tryptophan enhancing agent effective to increase T cell-mediated cytolysis of the cancer cell. Preferred tryptophan enhancing agents for use in these methods are set forth above.

- 5 In still another aspect of the invention, methods for determining a condition characterized by the ability of cancer cells to resist or evade T cell-mediated cytolysis are provided. The methods include monitoring a sample of cancer cells from a patient who has or is suspected of having the condition for constitutive expression of indoleamine 2,3-dioxygenase, as a determination of the condition. Additional methods for determining
10 whether to treat a cancer patient with an inhibitor of indoleamine 2,3-dioxygenase also are provided. The methods include determining the expression of indoleamine 2,3-dioxygenase by the cancer cells of the patient; the expression of indoleamine 2,3-dioxygenase by the cancer cells determines that the patient can be treated with an inhibitor of indoleamine 2,3-dioxygenase to increase the susceptibility of the cancer cells to T cell attack. In the foregoing
15 methods, expression can be monitored by any method, including measuring the amount of IDO protein (e.g., by immunoassay methods, by measuring IDO enzymatic activity) or nucleic acid encoding the protein (e.g., by nucleic acid hybridization or amplification) that is expressed by the cancer cells.

- The invention also provides pharmaceutical preparations containing any one or more
20 of the compositions described herein. Such pharmaceutical preparations can include pharmaceutically acceptable diluents, carriers or excipients. The use of such compositions in the preparation of medicaments, particularly medicaments for the treatment of cancer and for increasing T cell proliferation also is provided.

- These and other aspects of the invention, as well as various advantages and utilities
25 will be more apparent with reference to the drawings and detailed description of the invention.

Brief Description of the Figures

Fig. 1 shows the growth in culture of CTL clone MZ2-CTL-82/30 with or without the addition of l-methyl-tryptophan.

30 Fig. 2 shows the growth in culture of CTL clone CTL-361A/17 with or without the addition of l-methyl-tryptophan.

Fig. 3 shows the growth in culture of CTL clone CTL-361A/21 with or without the addition of l-methyl-tryptophan.

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Figs. 4A, 4B and 4C depict the lytic activity of CTL clones MZ2-CTL-82/30, CTL-361A/17 and CTL-361A/21 respectively on the autologous tumor cells (-●-) or NK-target cells K562 (-Δ-) after culture with or without the addition of 1-methyl-tryptophan. The autologous tumor cells were melanoma cells MZ2-MEL-43 in the case of CTL 82/30 and renal carcinoma cells LE9211-RCC in the case of CTLs 361A/17 and 361A/21.

Fig. 5 shows the tumor size and growth rate of tumors for groups of animals treated either with the IDO inhibitor 1-methyl tryptophan or PBS.

Detailed Description of the Invention

The culture of T cells *in vitro* has generally been a difficult procedure as compared to the culture of other cell types, e.g., fibroblasts, which readily grow in culture. Although several of the growth factors and nutrients which are required for T cell culture are now known, the culture of T cells still is not an easy or efficient task. The problems of T cell culture include slow rates of proliferation, limited numbers of divisions, and the inability of some T cell clones to grow in culture. "T cell" or "T cells," as used herein, refers to T lymphocytes including CD8⁺ T lymphocytes and CD4⁺ T lymphocytes.

A recent report by Munn et al. (*Science* 281:1191-1193, 1998) demonstrated that *in vivo* administration to pregnant mice of an inhibitor of indoleamine 2,3-dioxygenase (IDO; EC 1.13.11.17) led to the rejection of allogeneic but not syngeneic embryos. IDO is an enzyme responsible for the first step of tryptophan catabolism, which is the oxygenation of tryptophan into N¹-formyl-kynurenine. This enzyme is expressed at very high levels in placenta. The report suggested that IDO activity in placental cells suppressed cytolytic T lymphocyte responses, thereby permitting gestation of allogeneic embryos. When IDO activity of the placental cells was inhibited, cytolytic T lymphocyte responses were no longer inhibited and allogeneic embryos were rejected.

Based on their unpublished work on immunosuppressive macrophages that inhibit T cell proliferation by degrading tryptophan reported in the same paper, Munn et al. suggest that macrophages, by expressing high levels of IDO, locally deplete tryptophan thereby "paralyzing" T cells. The work of Munn et al. suggests that placental trophoblasts constitutively express high levels of IDO, and macrophages can be induced to express high levels of IDO, and thus these two cell types are responsible for suppressing T cell proliferation.

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Surprisingly, it has now been determined that inhibition of indoleamine 2,3-dioxygenase by 1-methyl-DL-tryptophan increased the proliferation of T cells in *in vitro* cultures which lack these IDO expressing cell types (i.e., placental trophoblasts and macrophages). This finding was unexpected for the reasons that (1) the T cell cultures that were used did not include trophoblasts or macrophages, (2) it is believed that none of the cells in those cultures expressed IDO over the course of the culture (in contrast to the high levels of IDO expression by trophoblasts and macrophages) and (3) any tumor cells that do express IDO in the culture are quickly lysed by the activated T cells.

In addition, a second unexpected finding is that certain tumors express IDO constitutively, as shown in the Examples below. In contrast to the observations of Munn which found constitutive IDO expression in placental trophoblasts and interferon- γ (IFN γ) inducible expression in other cells (such as macrophages), it was unexpectedly found that tumor cells also express IDO constitutively, without any requirement for IFN γ stimulation. In published PCT application WO99/29310, Munn et al predicted that T cell-derived IFN γ would be the signal for tryptophan degradation (see Example 4), but found that full induction of tryptophan degradation required a CD40L co-signal.

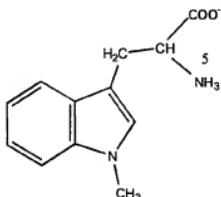
The compound 1-methyl-DL-tryptophan is a tryptophan enhancing agent that increases T cell proliferation. As used herein, a "tryptophan enhancing agent" is a compound or a molecule added to a cell or cell culture that increases the concentration of tryptophan in a cell culture and increases proliferation of cells in the cell culture in the absence of macrophages or trophoblasts. Exemplary tryptophan enhancing agents include IDO inhibitors (e.g., competitive inhibitors of IDO such as 1-methyl-DL-tryptophan, antisense nucleic acids that inhibit expression of IDO, anti-IDO antibodies that inhibit IDO activity) and compounds which directly increase tryptophan (e.g., tryptophan and analogs thereof which can be metabolized by T cells).

Assays to measure IDO enzymatic activity have been described (Cady and Sono, *Arch. Biochem. Biophys.* 292:326-333, 1991). Such methods can be used as screening assays to determine whether a compound inhibits the enzymatic function of IDO *in vitro*, i.e., whether a compound is an IDO inhibitor.

Additional analogs of tryptophan are also useful for increasing proliferation of T cells *in vitro*. The compound 1-methyl-DL-tryptophan and two other tryptophan analogs (β -(3-benzofuranyl)-DL-alanine and β -[3-benzo(*b*)thienyl]-DL-alanine were described as competitive

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inhibitors of indoleamine 2,3-dioxygenase by Cady and Sono. The structure of 1-methyl-tryptophan is given below:



10

Small molecule IDO inhibitors, such as those described above, are preferred. Other small molecule IDO inhibitors include 6-chloro-DL-tryptophan (Saito et al., *Neurosci. Lett.* 178(2):211-215, 1994), nitric oxide (NO) gas or the NO-generating compound, diethylamine dinitric oxide adduct (Thomas et al., *J. Biol. Chem.* 269(20):14457-14464, 1994), norharmane and 4-phenylimidazole (Sono et al., *Biochemistry* 28(13):5392-9; 1989) and 2,5-dihydro-L-phenylalanine (Watanabe et al., *Biochem. Biophys. Res. Commun.* 85(1):273-9, 1978). Prostaglandin inhibitors that inhibit the induction of IDO by interferon- γ , such as indomethacin, phenylbutazone and aspirin (Sayama et al., *Proc. Natl. Acad. Sci. USA* 78(12):7327-30, 1981) also may be used as IDO inhibitors. Inhibitors of tryptophan 2,3-dioxygenase (EC 1.13.11.11) such as 540C91 [(E)-3-[2-(4'-pyridyl)-vinyl]-1H-indole] (Reinhard et al., *Biochem Pharmacol.* 51(2):159-63, 1996) and 680C91 [(E)-6-fluoro-3-[2-(3-pyridyl)vinyl]-1H-indole] (Salter et al., *Biochem. Pharmacol.* 49(10):1435-42, 1995) also may be used as tryptophan enhancing agents.

Additional IDO inhibitors are isolated polypeptides which selectively bind IDO and reduce its activity. Isolated binding polypeptides include antibodies and fragments of antibodies (e.g. Fab, F(ab)₂, Fd and antibody fragments which include a CDR3 region which binds selectively to IDO). Preferred isolated binding polypeptides are those that bind to an epitope that is at or near the catalytic site of IDO.

The invention, therefore, in some embodiments involves the use of antibodies or fragments of antibodies which have the ability to selectively bind to IDO and stimulate T cell proliferation and/or activity under the conditions disclosed herein. Antibodies include polyclonal and monoclonal antibodies, prepared according to conventional methodology.

Significantly, as is well-known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W.R. (1986) *The Experimental Foundations of Modern Immunology* Wiley & Sons, Inc., New York; Roitt, I. (1991) *Essential Immunology*, 7th Ed., Blackwell Scientific Publications, Oxford). The pFc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an F(ab')₂ fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, in general, Clark, 1986; Roitt, 1991). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3). The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, are largely responsible for antibody specificity.

It is now well-established in the art that the non-CDR regions of a mammalian antibody may be replaced with similar regions of conspecific or heterospecific antibodies while retaining the epitopic specificity of the original antibody. This is most clearly manifested in the development and use of "chimeric" or "humanized" antibodies in which non-human variable regions or CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody. See, e.g., U.S. patents 4,816,567, 5,225,539, 5,585,089, 5,693,762 and 5,859,205.

Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for F(ab')₂, Fab, Fv and Fd fragments; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by

homologous human or non-human sequences; chimeric F(ab')₂ fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by

5 homologous human or non-human sequences; and chimeric Fd fragment antibodies in which the FR and/or CDR1 and/or CDR2 regions have been replaced by homologous human or non-human sequences. The present invention also includes so-called single chain antibodies.

Thus, the invention involves polypeptides of numerous size and type that bind specifically to IDO and inhibit its functional activity. These polypeptides may be derived also from sources

10 other than antibody technology. For example, such polypeptide binding agents can be provided by degenerate peptide libraries which can be readily prepared in solution, in immobilized form or as phage display libraries. Combinatorial libraries also can be synthesized of peptides containing one or more amino acids. Libraries further can be synthesized of peptides and non-peptide synthetic moieties.

15 Other antibodies that bind IDO inducers such as interferon- γ also are useful as IDO inhibitors in accordance with the invention.

Still other tryptophan enhancing agents include antisense nucleic acids. Antisense nucleic acids include short oligonucleotides as well as longer nucleic acids. Preferably the antisense nucleic acids are complementary to and bind to portions of the IDO coding sequence 20 or 5' untranslated sequence, thereby inhibiting translation of functional IDO protein. Other antisense nucleic acids which reduce or block IDO transcription are also useful as tryptophan enhancing agents.

Thus the invention embraces antisense oligonucleotides that selectively bind to a nucleic acid molecule encoding IDO, to reduce the expression (transcription or translation) of 25 IDO. As used herein, the term "antisense oligonucleotide" describes an oligonucleotide that is an oligoribonucleotide, oligodeoxyribonucleotide, modified oligoribonucleotide, or modified oligodeoxyribonucleotide which hybridizes under physiological conditions to DNA comprising a particular IDO gene (e.g., human IDO such as the cDNA sequences referenced in GenBank accession numbers M34455, AH002828, or X17668) or to an mRNA transcript 30 of that gene and, thereby, inhibits the transcription of that gene and/or the translation of that mRNA.

Those skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity with its target will depend upon the

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specific target selected, including the sequence of the target and the particular bases which comprise that sequence. It is preferred that the antisense oligonucleotide be constructed and arranged so as to bind selectively with the target under physiological conditions, i.e., to hybridize substantially more to the target sequence than to any other sequence in the target cell under physiological conditions. Based upon the sequences of IDO nucleic acids, including allelic or homologous genomic and/or cDNA sequences, one of skill in the art can easily choose and synthesize any of a number of appropriate antisense molecules for use in accordance with the present invention. In order to be sufficiently selective and potent for inhibition, such antisense oligonucleotides should comprise at least 10 and, more preferably, at least 15 consecutive bases which are complementary to the target, although in certain cases modified oligonucleotides as short as 7 bases in length have been used successfully as antisense oligonucleotides (Wagner et al., *Nature Biotechnol.* 14:840-844, 1996). Most preferably, the antisense oligonucleotides comprise a complementary sequence of 20-30 bases.

Although oligonucleotides may be chosen which are antisense to any region of the gene or mRNA transcripts, in preferred embodiments the antisense oligonucleotides correspond to N-terminal or 5' upstream sites such as translation initiation, transcription initiation or promoter sites. In addition, 3'-untranslated regions may be targeted. Targeting to mRNA splicing sites has also been used in the art but may be less preferred if alternative mRNA splicing occurs. In addition, the antisense is targeted, preferably, to sites in which mRNA secondary structure is not expected (see, e.g., Sainio et al., *Cell Mol. Neurobiol.* 14(5):439-457, 1994) and at which proteins are not expected to bind. Finally, although the IDO sequences referenced above are cDNA sequences, one of ordinary skill in the art may easily derive the genomic DNA corresponding to those cDNAs. Thus, the present invention also provides for antisense oligonucleotides which are complementary to the genomic DNA corresponding to nucleic acids encoding IDO. Similarly, antisense to allelic or homologous cDNAs and genomic DNAs are enabled without undue experimentation.

In one set of embodiments, the antisense oligonucleotides of the invention may be composed of "natural" deoxyribonucleotides, ribonucleotides, or any combination thereof. That is, the 5' end of one native nucleotide and the 3' end of another native nucleotide may be covalently linked, as in natural systems, via a phosphodiester internucleoside linkage. These oligonucleotides may be prepared by art recognized methods which may be carried out

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manually or by an automated synthesizer. They also may be produced recombinantly by vectors.

In preferred embodiments, however, the antisense oligonucleotides of the invention also may include "modified" oligonucleotides. That is, the oligonucleotides may be modified 5 in a number of ways which do not prevent them from hybridizing to their target but which enhance their stability or targeting or which otherwise enhance their therapeutic effectiveness.

The term "modified oligonucleotide" as used herein describes an oligonucleotide in which (1) at least two of its nucleotides are covalently linked via a synthetic internucleoside linkage (i.e., a linkage other than a phosphodiester linkage between the 5' end of one 10 nucleotide and the 3' end of another nucleotide) and/or (2) a chemical group not normally associated with nucleic acids has been covalently attached to the oligonucleotide. Preferred synthetic internucleoside linkages are phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidates, carboxymethyl esters and peptides.

15 The term "modified oligonucleotide" also encompasses oligonucleotides with a covalently modified base and/or sugar. For example, modified oligonucleotides include oligonucleotides having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified oligonucleotides may include a 2'-O-20 alkylated ribose group. In addition, modified oligonucleotides may include sugars such as arabinose instead of ribose. The present invention, thus, contemplates *in vitro* use of IDO antisense molecules as well as *in vivo* pharmaceutical preparations containing modified antisense molecules that are complementary to and hybridizable with, under physiological conditions, nucleic acids encoding IDO enzyme, together with pharmaceutically acceptable 25 carriers.

In another embodiment, the antisense nucleic acids of the invention may be produced by expression in cells by expression vectors introduced therein. The choice and design of an appropriate vector is within the ability and discretion of one of ordinary skill in the art.

Expression vectors containing all the necessary elements for expression are 30 commercially available and known to those skilled in the art. See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. According to this embodiment, cells are genetically engineered by the introduction into the cells of heterologous DNA (RNA) encoding antisense IDO nucleic acid.

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The antisense IDO nucleic acid is placed under operable control of transcriptional elements to permit the expression of the antisense IDO nucleic acid in the host cell.

Preferred systems for mRNA expression in mammalian cells are those such as pRc/CMV (available from Invitrogen, Carlsbad, CA) that contain a selectable marker such as
5 a gene that confers G418 resistance (which facilitates the selection of stably transfected cell lines) and the human cytomegalovirus (CMV) enhancer-promoter sequences. Additionally, suitable for expression in primate or canine cell lines is the pCEP4 vector (Invitrogen), which contains an Epstein Barr Virus (EBV) origin of replication, facilitating the maintenance of plasmid as a multicopy extrachromosomal element. Another expression vector is the pEF-
10 BOS plasmid containing the promoter of polypeptide Elongation Factor 1 α , which stimulates efficiently transcription *in vitro*. The plasmid is described by Mishizuma and Nagata (*Nuc. Acids Res.* 18:5322, 1990), and its use in transfection experiments is disclosed by, for example, Demoulin (*Mol. Cell. Biol.* 16:4710-4716, 1996). Additional vectors for delivery of antisense IDO nucleic acid will be known to one of ordinary skill in the art.

15 Various techniques may be employed for introducing antisense IDO nucleic acids into cells in accordance with the invention, depending on whether the nucleic acids are introduced *in vitro* or *in vivo* in a host. Such techniques include transfection of nucleic acid-CaPO₄ precipitates, transfection of nucleic acids associated with DEAE, transfection or infection with viruses including the nucleic acid of interest, liposome mediated transfection, and the like.

20 For certain uses, it is preferred to target the nucleic acid to particular cells. In such instances, a vehicle used for delivering a nucleic acid of the invention into a cell (e.g., a retrovirus, adenovirus or other virus; a liposome) can have a targeting molecule attached thereto. For example, a molecule such as an antibody specific for a surface membrane protein on the target cell or a ligand for a receptor on the target cell can be bound to or incorporated within the
25 nucleic acid delivery vehicle. Where liposomes are employed to deliver the nucleic acids of the invention, proteins which bind to a surface membrane protein associated with endocytosis may be incorporated into the liposome formulation for targeting and/or to facilitate uptake. Such proteins include capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half life, and the like. Polymeric delivery systems also have been used successfully to deliver nucleic acids into cells, as is known by those skilled in the art. Such systems even permit oral delivery of nucleic acids.

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- Expansion of T cells can be carried out in a variety of different culture vessels and under different culture conditions. In general, the same culturing conditions that are used for culturing T cells using the prior art methods are used herein, with the exception that one or more IDO inhibitors (and/or tryptophan) are added to the culture. An exemplary protocol for 5 culturing T cells according to the invention is provided in the Examples below. Other standard T cell culture protocols which differ in components or conditions (e.g., serum-free culture, addition of different cytokines, growth factors or nutrients, etc.) also can be modified in accordance with the invention.

Other types of cells in addition to T cells can benefit from being grown in the presence 10 of tryptophan enhancing agents, e.g. in growth medium. Most growth media contain fixed amounts of tryptophan as an essential amino acid for cell metabolism. As described herein, T cells have an unexpected requirement for additional tryptophan for increased proliferation, which can be provided in growth medium by adding tryptophan to the medium or, as shown herein, by inhibiting enzymes that catabolize tryptophan. The proliferation of many kinds of 15 cells that cannot synthesize tryptophan will benefit from the inclusion of tryptophan enhancing agents in growth media in addition to the standard nutrients which are well known in the art.

Increasing the proliferation of cells *in vitro*, as used herein, means increasing the number of cells by at least about 10% relative to the number of cells that are present in a 20 parallel control population of cells that are subjected to the same conditions as the IDO inhibitor-treated population with the exception that such control population is not contacted with the IDO inhibitors. Preferably, the number of cells are increased at least about 50%, more preferably 2-fold, yet more preferably 4-fold, still more preferably 10-fold and, most preferably, at least 20-fold relative to the number of cells that are present in the parallel 25 control population. It will be seen from the Examples that the increase in T cell numbers cultured according to the invention can be even greater (see, e.g., Figure 2B, growth curve of CTL-361A/17).

Increasing the proliferation of cells *in vivo* similarly means increasing the number of a particular kind of cells in the body, by similar amounts as above. For example, cytolytic T 30 cells that recognize a cancer associated antigen can be administered to a subject for the treatment of cancer cells that express the cancer associated antigen. According to the invention, the *in vivo* proliferation of the cytolytic T cells can be increased by the coadministration of a tryptophan enhancing agent. The proliferation of T cells *in vivo* can be

determined, for example, by measuring T cells in the subject's peripheral blood. Additional methods to measuring T cell proliferation *in vivo* will be known to one of ordinary skill in the art.

The use of tryptophan enhancing agents to enhance T cell culture also can increase the effector properties of the T cells, e.g., cytolytic activity, at least for some T cell clones cultured in the presence of one or more tryptophan enhancing agents. As is shown in the Examples and depicted in Figs. 4A, 4B and 4C, the specific lysis of target tumor cells by certain cytolytic T cell clones was increased after culture in the presence of 1-methyl-DL-tryptophan. Thus the invention includes methods for increasing activity of T cells by culturing the T cells in the presence of an effective amount of tryptophan enhancing agents to increase the activity of the T cells. In these methods, T cell activity is increased at least about 10%, preferably at least about 25%, more preferably at least about 50%, and most preferably at least about 100%.

The time period in which the number of T cells are increased can be adjusted according to the needs of the person culturing the T cells, and can be, at least in part, a function of the cell type (e.g., T cell clone) and the specific culture conditions used (growth medium, serum, cytokines, culture vessel, etc.) and/or the desired outcome (e.g., increased proliferation, increased activity, etc.). In general, this time period ranges from about 7 days (for short term expansion of T cells) to several weeks.

Routine procedures known to those of ordinary skill in the art can be used to determine the number of cells in culture as a function of increasing incubation time of the cultured cells with the IDO inhibitor (and/or tryptophan). Typically, expansion of the T cells in culture (increase in cell number) is measured by counting the cell numbers according to standard methods, for example, determining the actual cell numbers using a hematocytometer or cell counter or measuring incorporation of a specific dye. T cells also can be labeled using specific labeled antibodies and counted using an automated devices such as a fluorescence activated cell sorter (FACS). Thus, the optimization of the particular growth conditions and selection of the amounts of tryptophan enhancing agent (i.e. one or more IDO inhibitors and/or tryptophan) that are necessary to achieve the above-noted fold increases in cell numbers (or increase in T cell activity) are determined using no more than routine experimentation. Such routine experimentation involves, for example, (i) varying the amount of a tryptophan enhancing agent at constant incubation time; (ii) varying the incubation time at constant amounts of tryptophan enhancing agent; (iii) applying the foregoing optimization

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- experiments to determine the particular conditions necessary to achieve a pre-selected fold increase in T cell number; and (iv) varying other factors including, for example, the identity or the state of the tryptophan enhancing agent (e.g., soluble or immobilized), to optimize the culture conditions to achieve the desired results. Similar routine experimental studies can be
5 carried out using animal models for optimization of *in vivo* use of tryptophan enhancing agents.

T cells that are grown in accordance with the invention can be used in a variety of *in vitro* and *in vivo* applications. For example, generating larger numbers of T cells will find application in the field of drug testing and for *in vitro* study of T cell biology. T cells cultured
10 according to the invention also can be used for therapeutic purposes *in vivo*. For example, T cells isolated from a subject can be cultured *in vitro* for expansion and eventual return to the subject. Some therapeutic approaches using T cells are premised on a response by a subject's immune system, leading to lysis of antigen presenting cells, such as cancer cells which present one or more cancer associated antigens. One such approach is the administration of
15 autologous cytolytic T lymphocytes (CTLs) specific to a complex of a cancer associated antigen and a MHC molecule (major histocompatibility complex; also referred to as HLA, human leukocyte antigen) to a subject with abnormal cells of the phenotype at issue. It is within the ability of one of ordinary skill in the art to develop such CTLs *in vitro* for use in therapeutic methods such as adoptive transfer (Greenberg, *J. Immunol.* 136(5): 1917, 1986;
20 Riddel et al., *Science* 257: 238, 1992; Lynch et al, *Eur. J. Immunol.* 21: 1403-1410, 1991; Kast et al., *Cell* 59: 603-614, 1989).

Specific production of CTL clones is well known in the art. An example of a method for T cell differentiation is presented in International Application number PCT/US96/05607. Generally, a sample of cells taken from a subject, such as blood cells, are contacted with a cell
25 presenting the complex and capable of provoking CTLs to proliferate (e.g., cancer cells, dendritic cells). The target cell can be a transfectant, such as a COS cell transfected with nucleic acids encoding an antigen and a HLA molecule capable of presenting the antigen. These transfectants present the desired HLA/antigen complex on their surface and, when combined with a CTL of interest, stimulate its proliferation. COS cells are widely available,
30 as are other suitable host cells. The clonally expanded autologous CTLs then are administered to the subject. Other methods for selecting antigen-specific CTL clones include the use of fluorogenic tetramers of MHC class I molecule/peptide complexes which are used to detect specific CTL clones (Altman et al., *Science* 274:94-96, 1996; Dunbar et al., *Curr.*

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Biol. 8:413-416, 1998). T cell clones that are specific for antigens expressed on cells infected by a pathogen also can be prepared and administered as described above.

Adoptive transfer or other methods premised on *in vitro* expansion of T cells are not the only forms of therapy that is available in accordance with the invention. CTLs can also be provoked *in vivo*, using a number of approaches. One approach is the use of non-proliferative cells expressing the complex.

Thus T cells expanded according to the invention can be used to "immunize" subjects or as "vaccines". As used herein, "immunization" or "vaccination" means increasing or activating an immune response against an antigen. It does not require elimination or

eradication of a condition but rather contemplates the clinically favorable enhancement of an immune response toward an antigen. Generally accepted animal models can be used for testing of immunization against cancer using a T cells expanded according to the invention. For example, human cancer cells can be introduced into a mouse to create a tumor, and T cells that were expanded in cultures including tryptophan enhancing agents by the methods described herein can be administered to the mouse. The effect on the cancer cells (e.g., reduction of tumor size) can be assessed as a measure of the effectiveness of the cancer associated antigen nucleic acid immunization. Methods for increasing an immune response with T cells, including formulation of a T cell composition and selection of doses, route of administration and the schedule of administration are well known in the art. The tests also can be performed in humans, where the end point can be to test for the presence of enhanced levels of circulating CTLs against cells bearing the antigen.

Thus it will be appreciated that one or more tryptophan enhancing agents can be administered as a component of an immune response modulation composition. As used herein, an immune response modulation composition is a composition administered to a

subject to increase an immune response mediated by T cells. The immune response modulation composition can include T cells, antigens (e.g., peptides, proteins), nucleic acids encoding antigens, etc. which stimulate an immune response. The T cell mediated immune response induced or increased by any of these immune response modulation composition will be favorably modulated by inclusion of one or more tryptophan enhancing agents as part of the immune response modulation composition.

Adjuvants also can be added to the immune response modulation compositions. Many kinds of adjuvants are well known in the art. Specific examples of adjuvants include monophosphoryl lipid A (MPL, SmithKline Beecham), a congener obtained after purification

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and acid hydrolysis of *Salmonella minnesota* Re 595 lipopolysaccharide; saponins including QS21 (SmithKline Beecham), a pure QA-21 saponin purified from *Quillja saponaria* extract; DQS21, described in PCT application WO96/33739 (SmithKline Beecham); combinations of DQS21/MPL mixed in ratios of about 1:10 to 10:1; QS-7, QS-17, QS-18, and QS-L1 (So et al., *Mol. Cells* 7:178-186, 1997); incomplete Freund's adjuvant; complete Freund's adjuvant; montanide; and various water-in-oil emulsions prepared from biodegradable oils such as squalene and/or tocopherol. Other adjuvants are known in the art and can be used in the invention (see, e.g. Goding, *Monoclonal Antibodies: Principles and Practice*, 2nd Ed., 1986). Methods for the preparation of mixtures or emulsions of antigens and adjuvant are well known to those of skill in the art of vaccination.

Other agents which stimulate the immune response of the subject can also be administered to the subject in an immune response modulation composition. For example, other cytokines are also useful in vaccination protocols as a result of their lymphocyte regulatory properties. Many other cytokines useful for such purposes will be known to one of ordinary skill in the art, including interleukin-12 (IL-12) which has been shown to enhance the protective effects of vaccines (see, e.g., *Science* 268: 1432-1434, 1995), GM-CSF and IL-18. Thus cytokines can be administered in conjunction with T cells and adjuvants to increase the immune response to the antigens.

There are a number of immune response potentiating compounds that can be used in vaccination protocols. These include costimulatory molecules provided in either protein or nucleic acid form. Such costimulatory molecules include the B7-1 and B7-2 (CD80 and CD86 respectively) molecules which are expressed on dendritic cells (DC) and interact with the CD28 molecule expressed on the T cell. Another costimulatory molecule is the ICOS protein. These interactions provide costimulation (signal 2) to an antigen/MHC/TCR (T cell receptor) stimulated (signal 1) T cell, increasing T cell proliferation and effector function.

As shown in the Examples, certain cancers unexpectedly have been found to constitutively express indoleamine 2,3-dioxygenase. These cancer cells would be expected to reduce the local concentration of tryptophan and disable T cell mediated immune responses to the cancer. The recognition of this unexpected property of cancer cells permits treatment of the cells, which may have evaded or have the potential to evade T cell-mediated cytolysis, to increase immune recognition and destruction of the cancer cells. The methods include administering to a subject in need of such treatment an amount of a tryptophan enhancing agent effective to increase T cell-mediated cytolysis of the cancer cell. Other methods include

contacting the cancer cells with an amount of a tryptophan enhancing agent effective to increase T cell-mediated cytosis of the cancer cell. Preferred tryptophan enhancing agents are described elsewhere herein.

The recognition that cancer cells can constitutively express indoleamine 2,3-

- 5 dioxygenase also permits one of ordinary skill in the art to determine a condition characterized by the ability of cancer cells to resist or evade T cell-mediated cytosis. For example, one can monitor a sample of cancer cells from a patient who has or is suspected of having the condition for constitutive expression of indoleamine 2,3-dioxygenase, as a determination of the condition. Once it is established that the subject has a cancer that
10 expresses indoleamine 2,3-dioxygenase, the skilled artisan can determine whether to treat the cancer patient with an inhibitor of indoleamine 2,3-dioxygenase. Expression of indoleamine 2,3-dioxygenase by the cancer cells indicates that the patient is a good candidate to be treated with an inhibitor of indoleamine 2,3-dioxygenase to increase the susceptibility of the cancer cells to T cell attack. In these methods, expression of indoleamine 2,3-dioxygenase by the
15 cancer cells of the patient can be monitored by any method, including measuring the amount of IDO protein or nucleic acid encoding the protein that is expressed by the cancer cells. IDO protein can be measured directly, such as by standard immunoassays, or indirectly, such as by measuring IDO enzymatic activity in accordance with known methods. IDO nucleic acid can be measured by nucleic acid hybridization or amplification, such as PCR.

20 When administered *in vivo*, the compositions of the present invention can be administered in pharmaceutically acceptable preparations. Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, supplementary immune potentiating agents such as adjuvants and cytokines and optionally other therapeutic agents.

25 The therapeutics of the invention such as tryptophan enhancing agents and/or T cells expanded *in vitro* in the presence of tryptophan enhancing agents can be administered *in vivo* by any conventional route, including injection or by gradual infusion over time. The administration may, for example, be oral, intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous, or transdermal. When antibodies are used therapeutically (e.g.,
30 antibodies that bind IDO), a preferred route of administration is by pulmonary aerosol. Techniques for preparing aerosol delivery systems containing antibodies are well known to those of skill in the art. When using antisense preparations of the invention, slow intravenous administration is preferred. Standard references in the art (e.g., *Remington's Pharmaceutical*

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Sciences, 18th edition, 1990) provide modes of administration and formulations for delivery of compounds.

The compositions of the invention are added to cell cultures in effective amounts or administered to a subject in effective amounts. An "effective amount" is that amount of a tryptophan enhancing agent composition that alone, or together with further doses, produces the desired response, e.g. increases proliferation and/or activity of T cells. This can be monitored by routine methods known to one of ordinary skill in the art.

The amount added to a cell culture *in vitro* may depend on the particular T cell clone and/or cell culture conditions used. The amounts administered *in vivo* may depend, of course, 10 the individual patient parameters including age, physical condition, size and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is generally preferred that a maximum dose of the 15 individual components or combinations thereof be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by 20 a different, more localized delivery route) may be employed to the extent that patient tolerance permits.

The compositions used in the foregoing methods preferably are sterile and contain an effective amount of one or more tryptophan enhancing agents for producing the desired response in a unit of weight or volume suitable for addition to a cell culture or administration 25 to a subject. As used herein, a subject is a human or non-human veterinary animal, including non-human primates, mice, rats, cows, pigs, horses, sheep, goats, dogs, cats, etc. Preferably the subject is a human.

When added to cell cultures, tryptophan enhancing agents are sterile and formulated for compatibility with cells, cell culture media and other cell culture components.

30 Sterilization may be carried out by filtration, irradiation or any other method compatible with cell culture components.

When administered to a subject, the pharmaceutical preparations of the invention are applied in pharmaceutically-acceptable amounts and in pharmaceutically-acceptable

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compositions. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be

- 5 pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically and pharmaceutically-acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the
10 like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts.

A tryptophan enhancing agent composition may be combined, if desired, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating
15 substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the
20 desired pharmaceutical efficacy.

The pharmaceutical compositions may contain suitable buffering agents, including: acetic acid in a salt; citric acid in a salt; boric acid in a salt; and phosphoric acid in a salt.

The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

25 The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely
30 divided solid carrier, or both, and then, if necessary, shaping the product. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed

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including synthetic mono-or di-glycerides. Carrier formulation suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA.

5

Examples

Example 1: Expression of IDO in tumor cells.

Indoleamine 2,3-dioxygenase (IDO) has been reported to be constitutively expressed only in placenta, and is inducible by IFN γ in many cells including tumor cells and 10 macrophages. No other reports of constitutive expression of IDO (i.e. without induction with IFN γ) are known.

IDO expression in a variety of mouse and human tumor cell lines was tested by RT-PCR in the absence of IFN γ to avoid induction of IDO expression by IFN γ . The tumor cell lines that were tested were tumor lines growing *in vitro*; the cells were not in contact with 15 IFN γ . The PCR reactions for mouse IDO expression were performed as described by Munn et al. (*Science* 281:1191-1193, 1998). For human tumor cells, PCR amplification primers were selected based on the human IDO nucleotide sequence (GenBank accession number M34455). The sense primer was 5'-ggt cat gga gat gtc cgt aa-3' (SEQ ID NO:1). The antisense primer was 5'-ctt tca cac agg cgt cat aa-3' (SEQ ID NO:2). The PCR was run as follows: 5 min at 20 94°C; 30 cycles of 1 min at 94°C, 2 min at 59°C and 3 min at 72°C; and a final elongation step of 15 min at 72°C. The size of the PCR product was 690 bp. As positive controls, expression of IDO was determined by PCR in placenta and in human tumor cells treated with IFN γ . High levels of expression were found in placenta and in induced tumor cells as expected according to previously published results.

25 Unexpectedly, two out of twelve mouse tumors were positive (one very strongly (leukemia WEHI-3B) the other moderately (mastocytoma P815)) and two out of twelve human tumor cell lines were moderately positive (1 melanoma, 1 renal cell carcinoma). Thus constitutive expression of IDO in a significant proportion of tumors was found unexpectedly, which represent the first cells known to express IDO constitutively outside of placenta. The 30 results of the examination of IDO expression in a larger set of human tumors is described in Example 3 below.

These results suggest that certain tumors express IDO and thereby are capable of paralysing the T cells that could otherwise attack them. These surprising results suggest that

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- IDO inhibitors could be used *in vivo* in patients bearing tumors that express IDO constitutively. This possibility was tested in mice bearing tumors and the results are presented below in Example 4. These patients are more likely to benefit from IDO inhibition in the framework of cancer immunotherapy, as their tumors, by expressing IDO
- 5 constitutively, are capable of downregulating the tumocidal effects of T cells which recognize the tumor. Therefore IDO inhibitors can be useful as a therapeutic agent for increasing the effectiveness of immunotherapy.

Example 2: Effect of IDO inhibitor on T cell proliferation and activity *in vitro*.

- 10 The effect of the indoleamine 2,3-dioxygenase (IDO) inhibitor, l-methyl-DL-tryptophan, was tested on T cell growth *in vitro*. l-methyl-DL-tryptophan (purchased from Sigma Chemical Co., St. Louis, MO) was added to three different cultures of human CTL, namely MZ2-CTL-82/30, which recognizes a MAGE-1 peptide presented by HLA-A1 (Traversari, *J. Exp. Med.*, 176:1453-1457, 1992), CTL 361A/17, which recognizes a RAGE-1
- 15 peptide presented by HLA-B7 (Gaugler et al., *Immunogenetics* 44(5):323-30, 1996), and CTL 361A/21, which recognizes a RUR-1 peptide presented by HLA-B7 (U.S. serial number 60/085,318). A 4mM stock solution of l-methyl-tryptophan was prepared in PBS containing 5mM HCl and was sterilized by irradiation. Fifty microliters of this solution were added (final concentration: 100 µM) to CTL cultures set up in 2 ml of Iscove's medium
- 20 supplemented with 10% pooled human serum, 50 U/ml interleukin-2, asparagine, arginine, glutamine and antibiotics according to routine practice. 5×10^5 CTL were seeded per well, together with 1×10^6 irradiated (100 Gy) feeder cells (LG2-EBV-B, an Epstein-Barr virus immortalized B cell line) and 1×10^4 irradiated autologous tumor cells. The autologous tumor cells were melanoma cells MZ2-MEL.43 in the case of MZ2-CTL-82/30, and renal carcinoma
- 25 cells LE9211-RCC in the case of CTL 361A/17 and 361A/21. Every week, the CTL were harvested, washed and restimulated in the same conditions with or without fresh inhibitor. CTL were counted weekly at the time of restimulation, and the cumulative growth curves calculated for each CTL clone are shown in Figs. 1, 2 and 3.

- As can be seen in the figures, addition of l-methyl-tryptophan induced a dramatic
- 30 increase in CTL proliferation, which was particularly observable after 2 or 3 weeks of culture (well after the lysis of any tumor cells in the culture by the CTLs). Two of the CTL clones, namely 82/30 and 361A/17, even stopped growing after 3 weeks in the absence of inhibitor, although they continued to grow exponentially with the inhibitor.

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- To determine whether the CTL treated with the inhibitor retained their activity and their specificity, a lysis assay was performed using as targets either autologous tumor cells or natural killer (NK)-target cells K562 (Figs. 4A, 4B and 4C). The test was performed after 4 weeks of culture with or without the inhibitor for CTL 82/30, and after 6 weeks of culture for 5 CTL 361A/17 and 361A/21. The data showed that the CTL treated with 1-methyl-tryptophan were still active and specific. The lytic activity of treated CTLs 82/30 and 361A/17 was even higher than that of the untreated ones.

From these data, it is clear that 1-methyl-tryptophan can be used to increase the proliferation of T cells *in vitro* without any loss of activity or specificity, and for certain 10 clones improves the activity (see Figs. 4A, 4B and 4C).

CTL 82/30, MZ2-MEL43 and LG2-EBV were tested for IDO expression by PCR and all were negative. LE-9211-RCC was also tested and is positive (although not as strong as placenta). However, the tumor cells quickly disappear in the culture since they are lysed by the CTL. Therefore there are possibly IDO expressing cells in the culture at the beginning, 15 but these are removed by lysis and therefore cannot account for the effect of the IDO inhibitor that was observed.

These results also demonstrate that IDO inhibitors such as 1-methyl-tryptophan can have effects in the absence of cells that constitutively express high levels of IDO, such as trophoblasts or macrophages, and outside of the placenta. Thus IDO inhibitors could also be 20 used *in vivo* as an adjuvant for improving T cell responses against antigens, including cancer associated antigens or antigens expressed on cells infected by a pathogen.

Example 3: Expression of indoleamine 2,3-dioxygenase by human tumors

Indoleamine 2, 3-dioxygenase (IDO) has been shown to inhibit proliferation of T cells 25 through the selective depletion of tryptophan, an amino acid that is absolutely required for T cell division. Constitutive expression of IDO by tumor cells may therefore prevent effective T cell responses against these tumors through tryptophan starvation.

A total of 268 human tumor lines were tested by PCR for the constitutive expression of human IDO. The PCR primers used for amplification were, as above, 30 5'-ggcatggagatgtccgtaa-3' (sense; SEQ ID NO:1) and 5'-cttcacacaggcgctataa-3' (antisense; SEQ ID NO:2). The DNA was denatured at 94°C for 5 min followed by 30 cycles of 94°C (1 min), 59°C (2 min), 72°C (3 min). The expected size for the amplified product was 690 bp. Table 1 summarizes the results of the amplifications, showing 29% (77/268) of all tumors

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tested constitutively express IDO. In some tumor groups the constitutive expression of IDO was found in 80% of the tumors tested.

TABLE 1: Constitutive Expression of IDO by Human Tumor Cell Lines

5

TUMOR CELL LINE	NO. EXPRESSING IDO/NO. TESTED
Thyroid	0/2
Choriocarcinoma	0/1
Colon-Rectal	2/17 (12%)
Stomach	0/2
Leukaemia	0/9 (0%)
Lymphoma	0/6 (0%)
Melanoma	29/109 (27%)
Ophthalmic Melanoma	0/1
Mesothelioma	13/19 (68%)
Myeloma	0/6 (0%)
Neuroblastoma	0/1
Head and Neck	12/15 (80%)
Ovarian	1/1
Pancreatic	4/5 (80%)
Non-Small Cell Lung	5/12 (42%)
Small Cell Lung	1/27 (3.7%)
Kidney	3/16 (19%)
Sarcoma	2/7 (29%)
Breast	1/4 (25%)
Thyroid	0/1
Uterine	0/1
Gall Bladder	1/2
Bladder	3/4 (75%)
TOTAL	77/268 (29%)

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Example 4: Treatment with IDO inhibitor retards *in vivo* growth of an IDO expressing murine tumor

Because T cell responses may be inhibited *in vivo* by tumors that express IDO, the effect on tumor growth by treating tumor-laden mice with the IDO inhibitor, 1-methyl-tryptophan, was investigated.

Three groups of 20 female Balb/c mice were injected subcutaneously in the left flank with either 100,000, 200,000 or 500,000 live cells of tumor line WEHI 3B which expresses high levels of the T cell inhibitor, IDO. On the day prior to tumor injection and on every second day following tumor injection, 10 mice from each of these groups underwent therapeutic treatment with an intraperitoneal injection of 10 mg of 1-methyl-tryptophan. The remaining 10 mice from each of these groups were treated with phosphate buffered saline (PBS) as a control. The size of the developing tumor on each mouse was measured every second day.

Fig. 5 summarizes the tumor size and growth rate over a period of 30 days for the three groups of animals treated either with 1-methyl-tryptophan or PBS. The data indicate that treatment with 1-methyl-tryptophan in the group of mice injected with 100,000 tumor cells retarded the development of the tumor compared to the rate of growth of tumors in mice treated with PBS. These results suggest that 1-methyl-tryptophan inhibits tumor growth and therefore may be useful in therapeutic treatment of tumors expressing IDO.

20

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

All references disclosed herein are incorporated by reference in their entirety.

We claim:

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Claims

1. A method for determining a condition characterized by the ability of cancer cells to resist or evade T cell-mediated cytolysis comprising

5 monitoring a sample of cancer cells from a patient who has or is suspected of having the condition for constitutive expression of indoleamine 2,3-dioxygenase, as a determination of the condition.

2. The method of claim 1, wherein the indoleamine 2,3-dioxygenase is monitored by measurement of indoleamine 2,3-dioxygenase nucleic acid expression.

10 3. The method of claim 2, wherein the indoleamine 2,3-dioxygenase nucleic acid expression is measured by nucleic acid hybridization or nucleic acid amplification.

15 4. The method of claim 1, wherein the indoleamine 2,3-dioxygenase is monitored by measurement of indoleamine 2,3-dioxygenase polypeptide expression.

5. The method of claim 4, wherein the indoleamine 2,3-dioxygenase polypeptide expression is measured by immunoassay.

20 6. The method of claim 4, wherein the indoleamine 2,3-dioxygenase polypeptide expression is measured by measuring IDO enzymatic activity.

7. A method for determining whether to treat a cancer patient with an inhibitor of indoleamine 2,3-dioxygenase, comprising

25 determining the constitutive expression of indoleamine 2,3-dioxygenase by the cancer cells of the patient, wherein the constitutive expression of indoleamine 2,3-dioxygenase determines that the patient will be treated with an inhibitor of indoleamine 2,3-dioxygenase.

30 8. A method for treating a subject having or suspected of having a tumor, the cells of which constitutively express indoleamine 2,3-dioxygenase, comprising administering to a subject in need of such treatment an amount of an indoleamine 2,3-dioxygenase inhibitor effective to inhibit the activity of the constitutively expressed indoleamine 2,3-dioxygenase.

9. A method for treating a cancer cell which has evaded or has the potential to evade T cell-mediated cytolysis, comprising

administering to a subject in need of such treatment an amount of a tryptophan

- 5 enhancing agent effective to increase T cell-mediated cytolysis of the cancer cell, wherein the cancer cell constitutively expresses indoleamine 2,3-dioxygenase.

10. A method for increasing proliferation of cells, comprising

growing the cells in the presence of an amount of one or more tryptophan enhancing

- 10 agents effective to increase the proliferation of the cells.

11. The method of claim 10, wherein the tryptophan enhancing agent is an inhibitor of indoleamine 2,3-dioxygenase.

- 15 12. The method of claim 11, wherein the inhibitor of indoleamine 2,3-dioxygenase is selected from the group consisting of 1-methyl-DL-tryptophan, β -(3-benzofuranyl)-DL-alanine and β -[3-benzo(*b*)thienyl]-DL-alanine.

13. The method of claim 12, wherein the inhibitor of indoleamine 2,3-dioxygenase is

- 20 1-methyl-DL-tryptophan.

14. The method of claim 10, wherein the tryptophan enhancing agent is tryptophan.

15. The method of claim 10, wherein proliferation of the cells is increased at least about

- 25 10% relative to a control population of cells.

16. The method of claim 15, wherein proliferation of the cells is increased at least about 50% relative to a control population of cells.

- 30 17. The method of claim 16, wherein proliferation of the cells is increased at least about 2-fold relative to a control population of cells.

18. The method of claim 10, wherein the cells are not macrophages or trophoblasts.

19. The method of any of claims 10-18, wherein the cells are T cells.
20. The method of claim 19, wherein the T cells are grown by proliferating *in vivo*.
5
21. The method of claims 19, wherein the T cells are grown by culturing *in vitro*.
22. The method of claim 20, wherein the activity of the T cells is increased.
- 10 23. The method of claim 22, wherein the activity of the T cells is cytolytic activity.
24. The method of claim 21, wherein the activity of the T cells is increased.
25. The method of claim 24, wherein the activity of the T cells is cytolytic activity.
15
26. An apparatus for culturing T cells, comprising a cell culture container containing a tryptophan enhancing agent and at least one T cell.
- 20 27. The apparatus of claim 26, wherein the tryptophan enhancing agent is an inhibitor of indoleamine 2,3-dioxygenase.
28. The apparatus of claim 26, wherein the cell culture container does not contain macrophages or trophoblasts.
- 25 29. The apparatus of claim 26, wherein the container further includes one or more growth media components for T cell culture.
30. A kit for stimulating the proliferation of T cells in the absence of cells expressing indoleamine 2,3-dioxygenase, comprising
30 a container containing a tryptophan enhancing agent and instructions for using the tryptophan enhancing agent to stimulate proliferation of T cells *in vitro* in the absence of macrophages and trophoblasts.

- 29 -

31. A growth medium for the culture of cells comprising a tryptophan enhancing agent.

32. The growth medium of claim 31, wherein the cells are T cells.

5 33. A T cell culture comprising at least one T cell, growth medium and a tryptophan enhancing agent.

34. The T cell culture of claim 33, wherein the culture does not contain macrophages or trophoblasts.

10

35. An immune response modulation composition comprising an amount of a tryptophan enhancing agent effective to increase local tryptophan concentrations in the presence of constitutively expressed indoleamine 2,3-dioxygenase and a pharmaceutically acceptable carrier.

15

36. The immune response modulation composition of claim 35, wherein the tryptophan enhancing agent is an inhibitor of indoleamine 2,3-dioxygenase.

20 37. The immune response modulation composition of claim 36, wherein the inhibitor of indoleamine 2,3-dioxygenase is 1-methyl-DL-tryptophan.

38. The immune response modulation composition of claim 35, further comprising one or more immune modulators selected from the group consisting of antigen-specific T lymphocytes, peptide antigens, antigenic proteins and nucleic acids encoding peptide 25 antigens.

39. The immune response modulation composition of claim 38, further comprising an adjuvant.

30 40. The immune response modulation composition of claim 39, wherein the adjuvant is selected from the group consisting of monophosphoryl lipid A (MPL); saponins including QS21, DQS21, QS-7, QS-17, QS-18, and QS-L1; DQS21/MPL; incomplete Freund's

- 30 -

adjuvant; complete Freund's adjuvant; montanide; and water-in-oil emulsions prepared from biodegradable oils.

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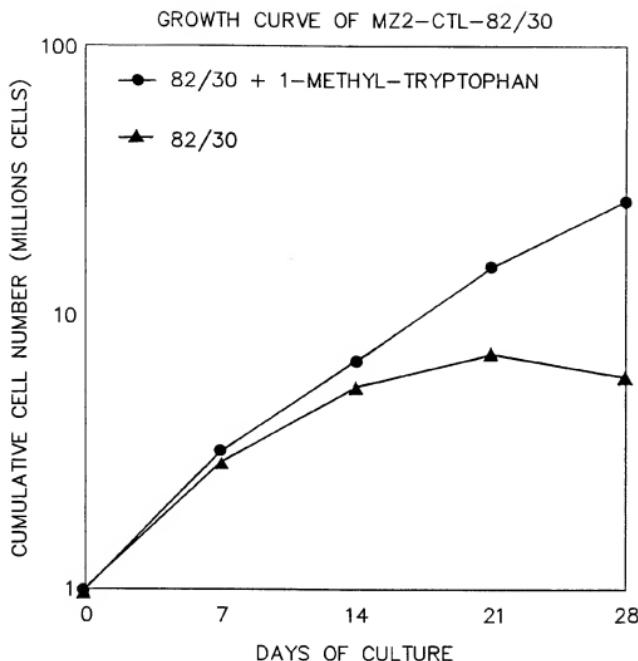


FIG. 1

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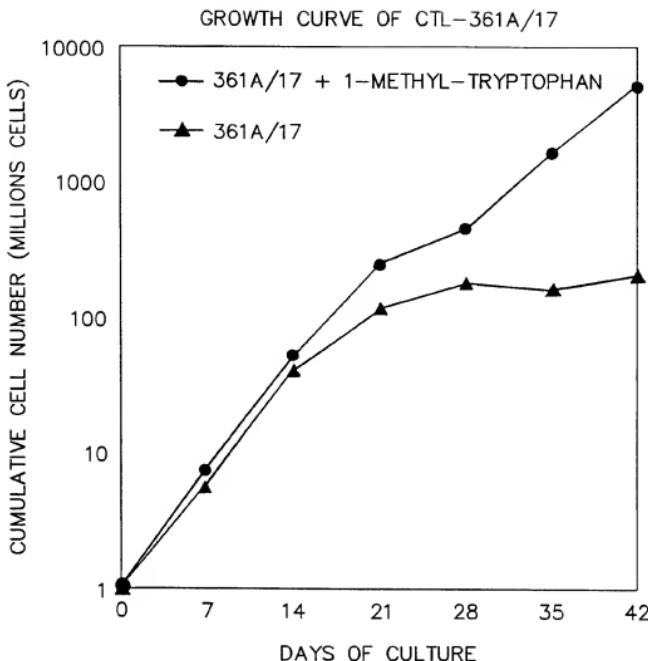


FIG. 2

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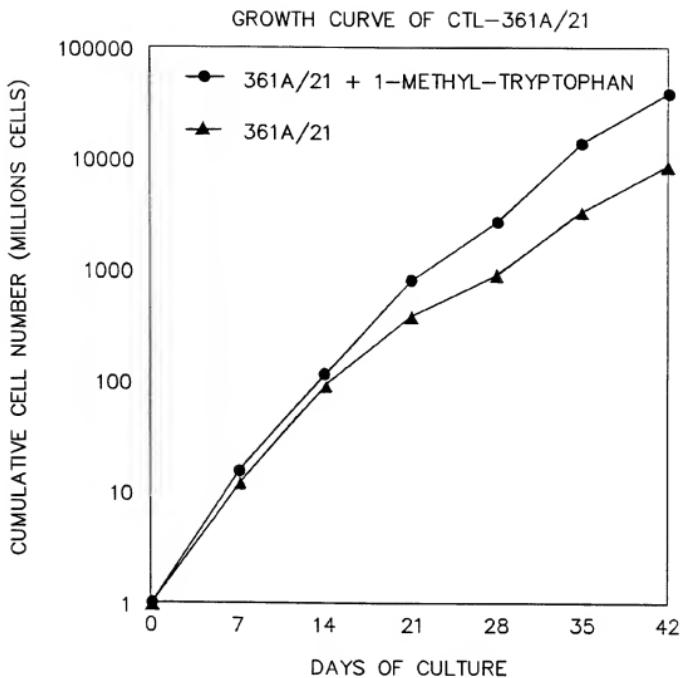


FIG. 3

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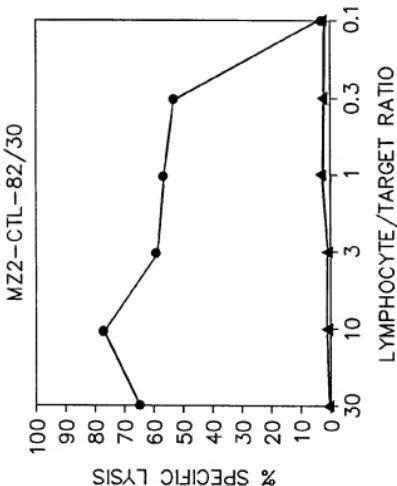
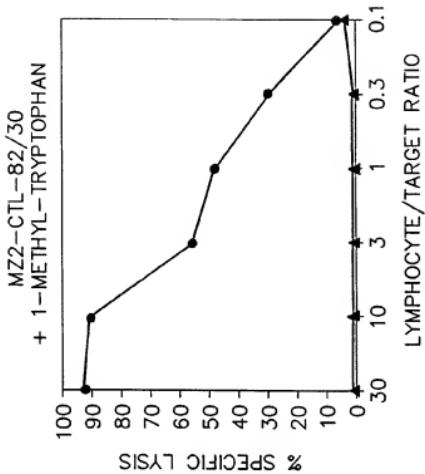


FIG. 4A

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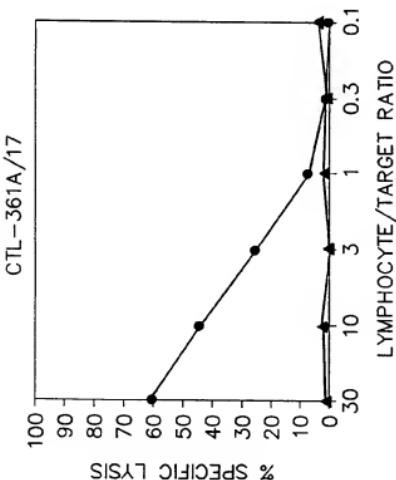
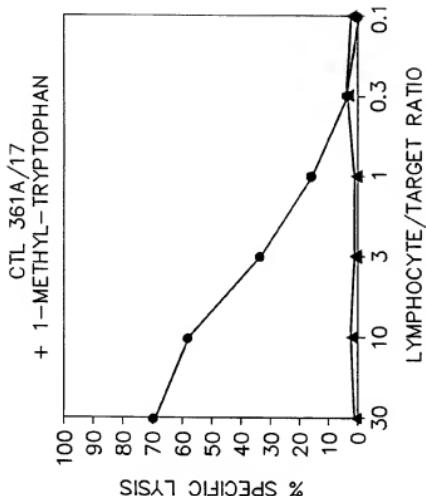


FIG. 4B

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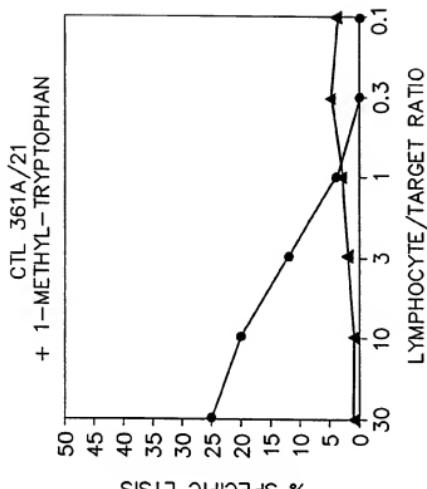
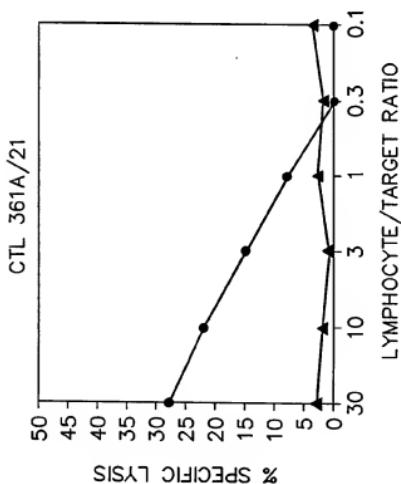


FIG. 4C



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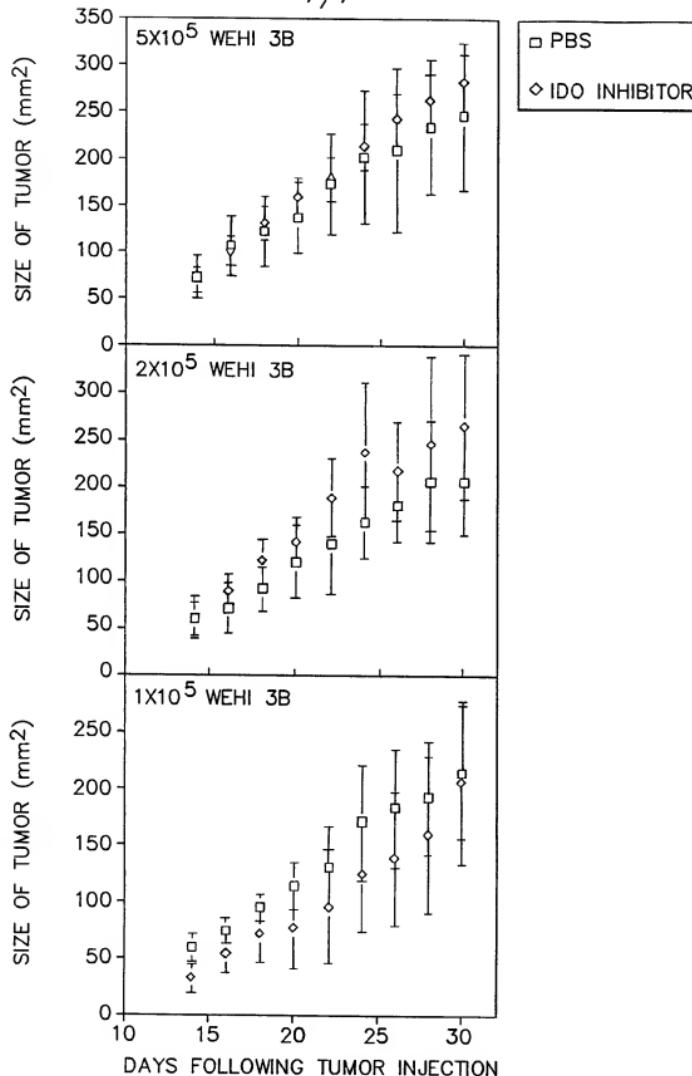


FIG. 5

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SEQUENCE LISTING

<110> Ludwig Institute for Cancer Research

5 <120> METHODS FOR INCREASING T CELL PROLIFERATION

<130> L0461/7060WO

10 <150> US 60/132,219

<151> 1999-05-03

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15 <170> FastSEQ for Windows Version 3.0

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/12118

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MUNN D.H. ET AL.: "Prevention of allogenic fetal rejection by tryptophan catabolism" SCIENCE, vol. 281, 1998, pages 1191-1193, XP002147184 cited in the application page 1193, column 1, line 9-15	1-40
X	MUNN D.H. ET AL.: "Regulation of T cell activation by macrophage (Mvariant phi)-mediated tryptophan (TRP) depletion" FASEB JOURNAL, vol. 12, 1998, page A276 XP002147185 abstract ---	1-40 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
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8 document member of the same patent family

Date of the actual completion of the international search

19 September 2000

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17/10/2000

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/12118

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MUNN D.H. ET AL.: "Inhibition of T cell proliferation by macrophage tryptophan catabolism" J. EXP. MED., vol. 189, 3 May 1999 (1999-05-03), pages 1363-1372, XP000938164 the whole document ---	1-40
X	MUNN D.H. ET AL.: "Macrophage inhibition of T cell activation via depletion of tryptophan." BLOOD, vol. 90, 1997, pages 448A-449A, XP000938340 abstract	1-40
P,X	WO 99 29310 A (MEDICAL COLLEGE OF GEORGIA RES) 17 June 1999 (1999-06-17) abstract claims	1-40
P,X	FRIBERG M.A. ET AL.: "Indoleamine 2,3-dioxygenase (IDO) protects established tumors from T cell mediated rejection." PROC. AMER. ASS. CANCER RES. ANN. MEET., vol. 41, March 2000 (2000-03), page 112 XP000946011 abstract	1-40
P,X	FRUMENTO G. ET AL.: "Inhibition of T cell proliferation by the purified enzyme indoleamine 2,3-dioxygenase" HUMAN IMMUNOLOGY, vol. 61, 2000, page S140 XP000938152 abstract	1-40
X	CADY ET AL: "1-Methyl-DL-tryptophan, beta-(3-benzofuranyl)-DL-alanine (the oxygen analog of tryptophan), and beta-'3-benzo(b)thienyl'-DL-alanine (the sulfur analog of tryptophan) are competitive inhibitors for indoleamine 2,3-dioxygenase" ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, US, NEW YORK, US, vol. 291, no. 2, December 1991 (1991-12), pages 326-333, XP002106396 ISSN: 0003-9861 abstract	1-40
	---	-/-

INTERNATIONAL SEARCH REPORT

Interinal Application No
PCT/US 00/12118

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHAPMAN ET AL: "Pharmacologically active benzo'b!thiophene derivatives. VIII. Benzo'b!thiophene analogs of tryptophan and .alpha.-methyltryptophan, and some of their 5-substituted derivatives" JOURNAL OF THE CHEMICAL SOCIETY, SECTION C: ORGANIC CHEMISTRY, GB, CHEMICAL SOCIETY. LETCHWORTH, no. 14, 1969, pages 1855-1858, XP002106399 abstract	1-40
X	PETERSON ET AL: "Evaluation of functionalized tryptophan derivatives and related compounds as competitive inhibitors of indoleamine 2,3-dioxygenase" MEDICINAL CHEMISTRY RESEARCH, US, BIRKHAEUSER, BOSTON, vol. 3, no. 8, 1994, pages 531-544, XP002106395 ISSN: 1054-2523 abstract	1-40
X	SOUTHAN ET AL: "Structural requirements of the competitive binding site of recombinant human indoleamine 2,3-dioxygenase" MEDICINAL CHEMISTRY RESEARCH, US, BIRKHAEUSER, BOSTON, vol. 6, no. 5, 1996, pages 343-352, XP002106394 ISSN: 1054-2523 abstract	1-40

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/12118

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9929310	A 17-06-1999	AU	1628599 A	28-06-1999
		AU	1628699 A	28-06-1999
		WO	9929852 A	17-06-1999